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ORIGINAL ARTICLE

Predictive values of vascular endothelial growth factor and microvessel-density levels in initial biopsy for prostate cancer

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Abstract Angiogenesis is an important factor in the development and progression of prostate cancer (PCA). We aimed to investigate the values of vascular-endothelial-growth-factor (VEGF) expression level and microvessel density (MVD) in the prediction of PCA diagnosis at repeated prostate biopsy (re-PBx). We retrospectively evaluated 167 patients with re-PBx according to elevated prostate-specific antigen levels, suspicious digital rectal examination, and the presence of premalignant lesions. Patients with PCA on re-PBx were included in the cancer group ($n = 17$). Patients with benign prostatic hyperplasia or normal tissues on re-PBx were included in the control group ($n = 21$). The groups were compared according to the expression level of VEGF and MVD in initial prostate biopsy. There was no statistically significant difference between groups according to age and serum prostate-specific-antigen values. The mean VEGF scores of the cancer and control groups were 232.64 ± 11.14 and 183.09 ± 14.56, respectively ($p < 0.05$). The mean MVD of the biopsy samples in the cancer and control groups were 246.47 ± 17.59 n/mm^2 and 197.33 ± 16.26 n/mm^2, respectively ($p < 0.05$). The cutoff values of VEGF scores and MVD were set as 200 and 215, respectively, for PCA detection in our study. Our results showed that the expression level of VEGF and MVD significantly increased in the initial prostate-biopsy samples of patients with PCA diagnosed with re-PBx. The evaluation of VEGF expression level and MVD might have an important value in the prediction of PCA at re-PBx. The expression level of VEGF and MVD should be kept in

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mind as PCA-related histopathological changes that indicate the increased angiogenesis in prostatic tissue.

Introduction

Prostate cancer (PCA) accounts for 10% of all cancers in men, and causes 9% of cancer-specific deaths in men in developed countries [1]. The incidence of PCA has increased with the development of new diagnostic tools. The measurement of prostate-specific antigen (PSA) and its derivatives, the increased frequency of prostate biopsy (PBx), and the specific diagnosis of premalignancy by biopsy in pathological investigations are the main factors resulting in repeat PBx (re-PBx). Histopathological signs on first PBx and PSA levels at follow-up are mainly used for the prediction of PCA diagnosis on re-PBx [2].

Angiogenesis is an important factor in the development and progression of PCA and other tumors. PCA cells secrete proangiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, interleukin-8, and platelet-derived growth factor [3].

Microvessel density (MVD) is the quantitative indicator of tumoral angiogenesis. Endothelium-specific antibodies, such as CD24, CD31, CD34, CD105, and von Willebrand factor (factor VIII), are used for the immunohistochemical staining of vessels, and MVD is calculated by counting the small and meandering vessels of the tumor. Recent investigations have suggested that increased MVD is related to high histological grade and poor prognosis in breast, lung, colon, stomach, malign melanoma, prostate, and bladder cancer [4,5].

Differences in VEGF expression level and MVD between PCA, premalignant lesions, and benign prostatic hyperplasia (BPH) have been reported. A pathological investigation of radical-prostatectomy materials showed an increased VEGF expression and MVD in PCA tissue in comparison to benign prostatic glands [6–8].

Here, we compared the VEGF expression level and MVD at re-PBx between patients initially diagnosed with BPH and PCA. We also investigated the values of VEGF expression level and MVD in the prediction of PCA diagnosis at re-PBx.

Methods

We retrospectively evaluated 1055 patients who underwent transrectal ultrasound (TRUS)-guided PBx between January 2000 and June 2013 at our department. A total of 167 patients underwent re-PBx during the study period due to an increased PSA level, suspicious digital rectal examination (DRE), or premalignant lesions, such as atypical small acinar proliferation (ASAP) and high-grade prostatic intraepithelial neoplasm (HGPIN). A total of 129 patients with ASAP, HGPIN, or prostatic inflammation were excluded. The remaining 38 patients were evaluated according to age, family history of PCA, laboratory findings, serum total PSA values, physical-examination findings, DRE findings, and radiological findings. The interval between the initial PBx and re-PBx was 6–12 months.

The patients were divided into the cancer and control groups. All patients in both groups were diagnosed with BPH on initial PBx. Seventeen patients with a PCA diagnosis at rebiopsy were included in the cancer group. The remaining 21 patients with a BPH diagnosis at re-PBx were included in the control group. These groups were compared according to VEGF score and MVD at initial PBx.

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Technique of TRUS-guided PBx

All patients were orally administered 500-mg ciprofloxacin twice daily for 2 days before undergoing PBx. The patient was placed in the left lateral decubitus position under local anesthesia for all TRUS evaluations and PBx procedures. A Logiq C2 ultrasound device with a transrectal probe (GE Healthcare, Milwaukee, WI, USA) was used to evaluate the prostate, and 5 mL of 2% prilocaine (2.5 mL for each side) was used as the local anesthetic agent and was injected bilaterally with a 22-gauge, 20-cm Chiba aspiration biopsy needle (GEOTEK Medical Corporation, Ankara, Turkey) just lateral to the junction between the prostate base and the seminal vesicle for periprostatic nerve blockade. The prostate was morphologically examined in transverse and sagittal planes after prilocaine infiltration. The ellipsoid formula was used to calculate the prostate volume. Five minutes after prilocaine injection, the PBx was performed in a transverse plane using the 10- to 12-core technique with a biopsy gun containing an 18-gauge, 25-cm Maxicore automatic biopsy gun needle (GEOTEK Medical Corporation).

Immunohistochemical staining and evaluation of VEGF expression and MVD levels

For all groups, hematoxylin/eosin-stained slides were reevaluated before immunohistochemical staining by the same pathologist. One core-biopsy sample of approximately 2 cm in diameter from each patient was used for immunohistochemical staining. Four-micrometer-wide paraffin blocks in polylysine-coated lam were deparaffinized in xylene and dehydrated in a series of baths containing decreasing concentrations of ethanol; then, the blocks were stained using the streptavidin–biotin–peroxidase method. Briefly, the sections were heated in citrate buffer (10mM, pH 6.0) at 120°C for 20–255 minutes (pressure cooker) for antigen retrieval, and were rinsed three times

in deionized distilled water. Endogenous peroxidase activity was blocked by 30 minutes of incubation with 0.3% hydrogen peroxidase. The sections were then incubated with the primary antibodies VEGF antibody (BioGenex, Fremont, CA, USA; immunogen: human recombinant VEGF165; clone: polyclonal; 1/100) and CD34 antibody (Thermo Scientific, Fremont, CA, USA; immunogen: detergent-solubilized vesicular suspension prepared from a perfusate of human term placenta; clone: QBend/10; 1/100) for 24 hours at room temperature. Then, the sections were stained according to the streptavidin—biotin method. The immunoreaction was visualized with 3-3′-diaminobenzidine/aminoethylcarbazole (Thermo Scientific Dako, Glostrup, Denmark) as a chromogen. The slides were counterstained with Mayer's hematoxylin solution and were mounted. All stages of staining were carried out at room temperature to avoid drying.

VEGF positivity was mostly detected in the epithelial cells of the prostate. Only 5% of the total VEGF staining was observed in fibroblasts. Therefore, we evaluated the VEGF expression according to epithelial staining. Cytoplasmic/membranous staining was considered positive for VEGF. The staining intensity was graded as grade 0 (absence of staining), grade 1 (mild staining), grade 2 (moderate staining), and grade 3 (severe staining). The VEGF score was calculated by multiplying the percent of stained epithelial cells in the biopsy sample with the grade of the VEGF staining. The VEGF score was used to make comparisons between the two groups.

All vessels in the biopsy samples with positive CD34 staining were counted. The vessels that had a lumen size large enough to contain eight erythrocytes were excluded. The vessels were evaluated under 40× magnification.

For MVD scoring, the most vascularized regions were selected at low magnification, and the microvessels were counted in three nonoverlapping areas at high-power magnification (400×). The mean value of the three MVD counts was considered the main score for MVD for each case. MVD is presented as the number of vessels/mm^2 (n/mm^2) for each group.

Statistical analysis

The data are expressed as the mean ± standard deviation. The demographic parameters, MVD values, and VEGF scores were compared between the two groups. SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. The contributions of the parameters were evaluated by the Levene test, and then the independent t test was used to compare the data between the two groups. Differences with a $p < 0.05$ were considered statistically significant.

Results

The mean ages of the cancer and control groups were 72.5 ± 7.5 years and 70.2 ± 6.6 years, respectively, and this difference was not statistically significant ($p = 0.324$). The mean blood-serum total PSA levels of the cancer and control groups were 11.46 ± 7.68 ng/mL and 10.81 ± 9.2 ng/mL, respectively, and that difference was also not statistically significant ($p = 0.258$). The Gleason scores of the patients in the cancer group were 6 in 13 (76.47%) patients, 7 in two (11.76%) patients, and 9 in one (5.88%) patient. Microscopically detectable cancer was found in only one (5.88%) patient in the cancer group.

VEGF

The mean percentages of VEGF staining in prostatic epithelial cells in the cancer and control groups were 92.35 ± 6.87% and 82.85 ± 21.18%, respectively, and the mean VEGF scores of the cancer and control groups were 232.64 ± 11.14 and 183.09 ± 14.56, respectively. The VEGF scores of the two groups were significantly different ($p = 0.013$) and are presented in Figure 1. The grade of VEGF staining of epithelial cells in the cancer group was higher than that in the control group, and these data are presented in Figures 2A and 2B. The cutoff value for the VEGF score was set at 200 for all patients, and had sensitivity, specificity, and positive and negative predictive values for PCA of 70.58%, 61.9%, 60%, and 72.2%, respectively. The area under the receiver-operating-characteristic curve was 0.71.

MVD

MVD was determined by counting the CD34-stained vessels of the biopsy samples, and the mean MVD values in the cancer and control groups were 246.47 ± 17.59 n/mm^2 and 197.33 ± 16.26 n/mm^2, respectively. The difference in MVD between the two groups was statistically significant ($p = 0.048$). The MVD values for both groups are presented in Figure 3. For both groups, areas with increased MVD are shown in Figures 4A and 4B. According to these results, the MVD values were higher in the cancer group than in the control group. The cutoff value for MVD was set at 215 for all patients, and had sensitivity, specificity, and positive and negative predictive values for PCA of 70.58%, 61.9%, 60%, and 72.2%, respectively. The area under the receiver-operating-characteristic curve was 0.718.

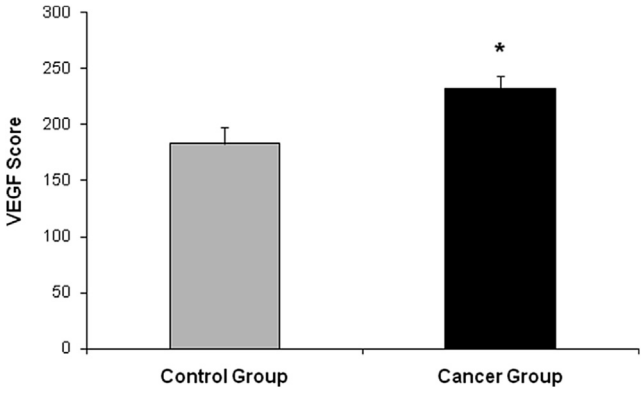

Figure 1. Distribution of vascular-endothelial-growth-factor scores in the control and cancer groups. The difference was significant between both groups (* $p = 0.013$). Data were expressed as mean ± standard deviation. VEGF = vascular endothelial growth factor.

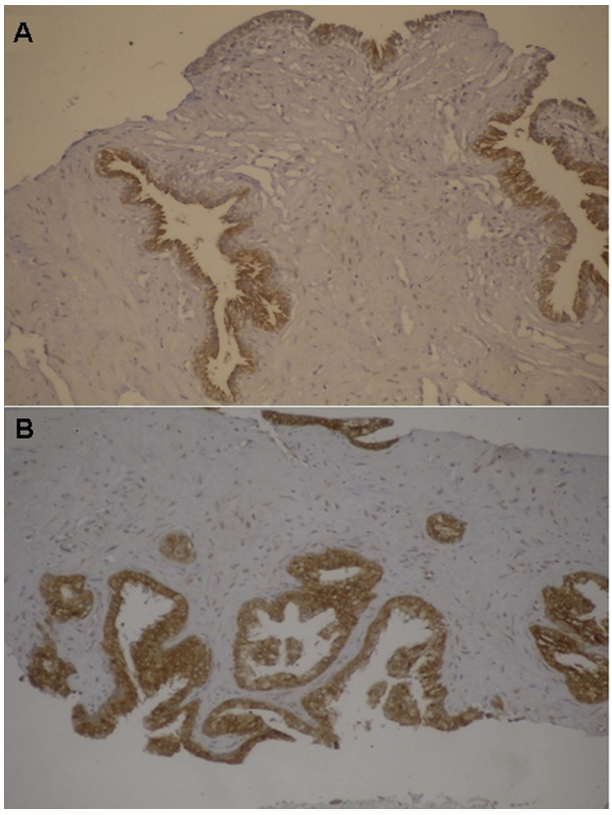

Figure 2. The vascular-endothelial-growth-factor expression in the epithelium of prostatic glands. (hematoxylin/eosin, 40×). (A) Low expression of vascular endothelial growth factor in the control group; (B) high expression of vascular endothelial growth factor in the cancer group.

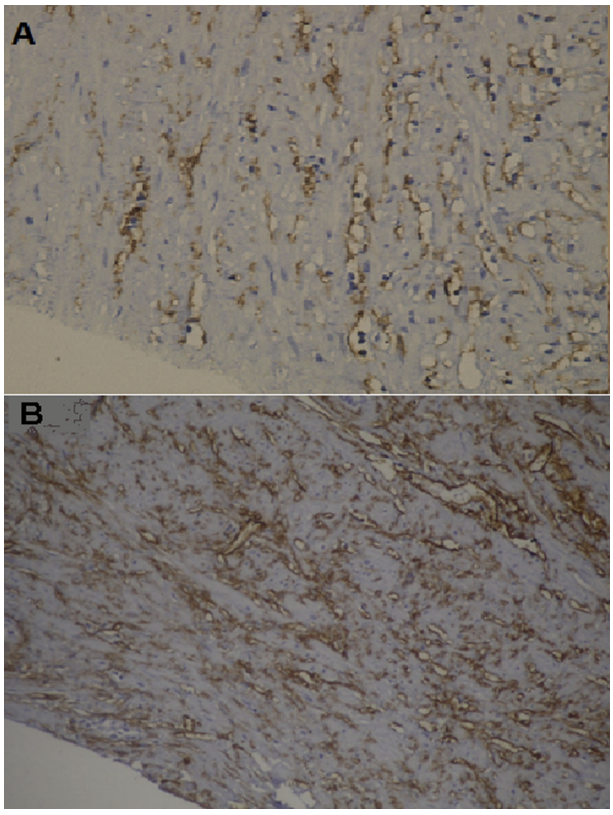

Figure 4. Microvessel density in prostatic tissue, determined by CD34 immunohistochemical staining. (A) Low density of microvessels in the control group (CD34, 40×); (B) high density of microvessels in the cancer group (CD34, 40×).

Discussion

The main factors for the prediction of PCA at re-PBx are the pathological findings of ASAP and HGPIN at initial PBx, and PSA elevation at the time of re-PBx [2]. Angiogenesis is important for the metastasis and growth of cancer. VEGF is a growth factor that stimulates angiogenesis, which contributes to tumor growth [9,10]. The quantitative marker of angiogenesis is MVD. For some tumors, a relationship between high MVD and poor prognosis has been reported [4,5]. Varying levels of MVD and VEGF expression in benign tissues and inflammatory tissues have also been shown by several studies [6—8]. We aimed to compare the VEGF scores and MVD values at initial PBx of patients diagnosed with BPH and PCA at re-PBx. We also aimed to investigate the values of VEGF level and MVD at initial PBx for the prediction of PCA.

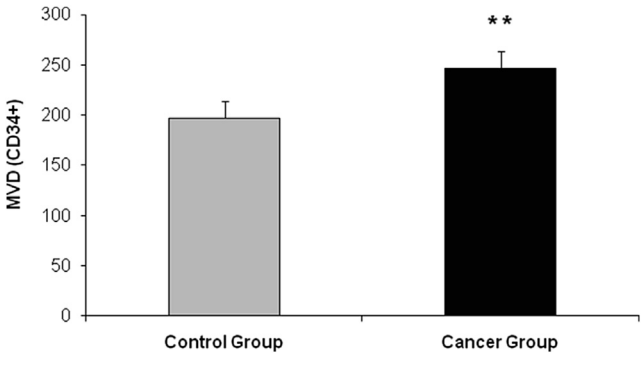

Figure 3. Distribution of microvessel density (CD34+) in the control and cancer groups. The difference was significant between both groups (** $p = 0.048$). Data were expressed as mean ± standard deviation. MVD = microvessel density.

Re-PBx is a required procedure in the presence of PCA suspicion. The main factors that influence the decision to perform re-PBx are the patient's symptoms, an elevated serum PSA level, signs suspicious of PCA on DRE, and premalignant lesions at initial PBx. Keetch et al. [11] reported a PCA risk of 25% in males with PSA levels of 4—20 ng/mL. The PCA detection rate of re-PBx is 20% in patients with a BPH diagnosis at initial PBx [11]. Djavan et al. [12] reported that two consecutive PBx procedures were effective to detect most clinically significant PCA cases among patients with PSA levels of 4—10 ng/mL. They suggested that a third and fourth PBx should only be applied for selected patients [12]. The reported cancer-detection rate of repeat TRUS-guided PBx for patients with HGPIN at initial PBx was 23% [13]. In contrast, for patients with ASAP at initial PBx, the PCA-detection rate of re-PBx was approximately 40—50%. The PCA region of the prostate in these patients has been reported to be similar to the ASAP region identified by

initial PBx [14,15]. Patients with ASAP and HGPIN at initial PBx were excluded from our study. Only patients with BPH or normal prostatic tissue at initial PBx were selected to investigate the importance of VEGF level and MVD in the prediction of PCA independent from ASAP and HGPIN.

Although the appropriate interval between two consecutive PBx procedures is controversial, the PCA incidence increases as that interval is increased [15]. The interval between the initial and re-PBx was 6—12 months in our study, and corresponded to the timing of the follow-up measurements of PSA level. Most patients in our study underwent repeat TRUS-guided PBx due to an elevated PSA level on consecutive measurements.

Understanding the molecular alterations in the early period of PCA is important. Angiogenesis is a critical stage of carcinogenesis. Angiogenesis is the main factor influencing the development and progression of PCA, and is related to a high Gleason score, metastasis, and poor prognosis [3]. The most important factor of angiogenesis is VEGF. Metastasis and mortality in PCA were reported to be related to VEGF expression [16]. The blood-plasma VEGF level was reported to be higher in patients with PCA and to serve as an independent prognostic factor for metastasis in men [17,18]. A study that independently investigated the pathological samples of 55 patients with radical prostatectomy showed that VEGF expression and MVD were significantly higher in the PCA region than the BPH, HGPIN, and normal prostatic-tissue regions in the same sample. Additionally, VEGF expression and MVD were reported to be higher in the BPH region than in the HGPIN region. The authors of that study suggested the existence of an upward trend in angiogenesis from BPH to premalignant to PCA tissues [6]. A similar study reported that MVD and VEGF expression were significantly higher in PCA than in benign tissues according to radical-prostatectomy pathology findings [7]. On the other hand, Wu et al. [8] did not find a significant difference in the expression of VEGF between tissues with benign prostatic epithelial cells and malignant tissues in patients who had undergone radical prostatectomy. In another study, the pathological samples of 72 patients who underwent transurethral resection of the prostate, radical prostatectomy, and TRUS-guided PBx were examined to determine the progression of PCA. Higher MVD values were reported in PCA patients with disease of higher clinic stage, and VEGF immunoreactivity was more intense in PCA regions than in benign tissues in the same samples [19]. The topography of neovascularization in PCA was evaluated in samples obtained from 14 patients who had undergone radical prostatectomy. Regions of BPH and PCA in the same samples were compared, and the MVD values of benign tissues were increased near to malignant tissue at radical prostatectomy [20]. Our hypothesis is supported by these studies. Our study is the first in the literature to compare the initial PBx pathologies of patients diagnosed with BPH and PCA by re-PBx. We investigated the probable roles of MVD and VEGF score in the prediction of PCA independent of ASAP and HGPIN, as the prognostic roles of these parameters are controversial. We found that the VEGF score and MVD at initial PBx were higher in patients diagnosed with PCA than in patients diagnosed with BPH at re-PBx. When the cutoff values of VEGF score and MVD were set at 200 and 215 for all patients, their sensitivity and specificity values for PCA detection were 70.58% and 61.9%, respectively. However, we believe that higher sensitivity and specificity values will be found by future investigations with larger sample sizes. Our findings showed that VEGF expression and MVD may be important markers for determining whether to perform re-PBx.

Lekas et al. [21] compared the MVD values of tissues in patients diagnosed with BPH and PCA after surgery. The immunoreactivity of CD34 in the hyperplasic glands of the prostate near the PCA was higher than that in other tissues. However, VEGF expression was not compared between BPH and benign tissues near the PCA in this study. The expression of VEGF was reported to be significantly increased in PCA tissues in comparison to BPH tissues [21]. We evaluated the initial PBx samples of patients diagnosed with BPH and PCA at re-PBx. We hypothesized that the initial PBx tissues of the cancer group were adjacent to the undiagnosed malignant tissue. Because of this hypothesis, we felt that our experimental design was valid for investigating the predictive roles of VEGF and MVD for PCA.

Angiogenesis is also related to inflammation in the prostate. MVD and VEGF expression have been shown to be affected by prostatic infections. The expressions of VEGF, cyclooxygenase-2, and B cell lymphoma 2 and MVD were studied in pathological samples of patients with PCA and BPH. Patients with BPH were divided into two groups: inflammatory BPH group and noninflammatory BPH group. The expression of VEGF in tissues in the inflammatory BPH group was higher than that in tissues of the noninflammatory BPH group and was lower than that in patients with PCA [22]. Patients with prostatic inflammation were excluded from our study to avoid the effect of inflammation on VEGF expression and MVD.

A limitation of our study was the small number of included patients. However, our patients were selected from a large series of patients who underwent PBx. Another limitation of this study was its retrospective nature.

We found that the angiogenetic factors VEGF score and MVD were increased at initial PBx in patients who were later diagnosed with PCA. The sensitivity and specificity values of VEGF score and MVD for the prediction of PCA were 70.58% and 61.9%, respectively. Our results suggest that VEGF expression and MVD might be important factors in deciding whether to perform re-PBx. Our study may lead to investigations of the carcinogenic parameters that have roles in the early period of PCA. There is no doubt that the findings of this study should be supported by the results of prospective randomized studies with a larger series of patients.

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